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Mutation in the Phosphorylation Sites of MAP Kinase Blocks Learning-Related Internalization of apCAM in *Aplysia* Sensory Neurons

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Summary

The synaptic growth that accompanies 5-HT-induced long-term facilitation of the sensory to motor neuron connection in *Aplysia* is associated with the internalization of apCAM at the surface membrane of the sensory neuron. We have now used epitope tags to examine the fate of each of the two apCAM isoforms (membrane bound and GPI-linked) and find that only the transmembrane form is internalized. This internalization can be blocked by overexpression of transmembrane constructs with a single point mutation in the two MAPK consensus sites, as well as by injection of a specific MAPK antagonist into sensory neurons. These data suggest MAPK phosphorylation at the membrane is important for the internalization of apCAMs and, thus, may represent an early regulatory step in the growth of new synaptic connections that accompanies long-term facilitation.

Introduction

Cellular studies of memory storage in the brain of both higher invertebrates and mammals suggest that the stabilization of long-term memory is achieved by the growth of new synaptic connections (Bailey and Kandel, 1993). Despite the association of structural changes with different forms of memory storage, surprisingly little is known about the intracellular signaling pathways and molecular mechanisms that convert neuronal activity into the formation of new and persistent synaptic connections.

To study these mechanisms, we have exploited the cellular specificity of a simple behavioral system, the gill-withdrawal reflex of *Aplysia*. This reflex can undergo both short- and long-term sensitization depending upon the pattern of tail stimulation. A single stimulus gives rise to short-term sensitization, an enhancement of the reflex response lasting minutes to hours, whereas five stimuli lead to long-term sensitization lasting 1 day or more. The short-term enhancement involves an alteration in the effectiveness of preexisting synaptic connections as a result of the covalent modification of preexisting proteins. The long-term form is associated with the growth of new synaptic connections initiated by a program of cAMP-inducible gene expression and the synthesis of new proteins (Bailey et al., 1996). Both the short- and long-term processes are represented on an elementary, cellular level in the connections between identified sensory neurons and motor neurons. The monosynaptic pathway can be reconstituted in dissociated cell culture, where 5-hydroxytryptamine (5-HT; serotonin), a modulatory neurotransmitter normally released by sensitizing stimuli, can substitute for the tail shock used during behavioral training in the intact animal (Montarolo et al., 1986). In parallel to behavioral sensitization (Frost et al., 1985), a single application of 5-HT produces short-term changes in synaptic effectiveness, whereas five spaced applications, or continuous exposure for 1.5 hr, produce long-term changes lasting 1 day or more.

About 20 proteins have now been identified that are modified by repeated pulses of 5-HT (Barzilai et al., 1989; Noel et al., 1993). Whereas the majority of these increase in their level of expression, five proteins are down-regulated by 5-HT. Four of these are immunoglobulin cell adhesion molecules related to the vertebrate neural cell adhesion molecule (NCAM) and to Fasciclin II in Drosophila (Mayford et al., 1992). Using gold-conjugated monoclonal antibodies to apCAM, Bailey et al. (1992) found that within 1 hr of the application of 5-HT or cAMP, a second messenger activated by 5-HT, there is also a decrease in the density of apCAM at the surface membrane of the sensory neuron. There is no comparable change in the postsynaptic cell. This presynaptic down-regulation of preexisting cell adhesion molecules is achieved by a rapid and transient activation of the endocytic pathway, leading to a protein synthesis-dependent internalization of apCAM and its rerouting from a pathway of apparent recycling to a pathway that seems destined for degradation. Concomitant with the down-regulation of apCAM, 5-HT and cAMP also induce in the sensory neurons an increase in the expression of the light chain of clathrin (apClathrin), one of the proteins whose expression is increased following long-term training, as well as an increase in the number of coated pits and coated vesicles (Hu et al., 1993). The 5-HT-induced decrease in apCAM is particularly prominent at sites where the processes of the sensory neurons contact one another, and is thought to have at least two major consequences: (1) disassembly of homophilically associated fascicles of the sensory neurons (defasciculation), a process that may destabilize adhesive contacts normally inhibiting growth; and (2) endocytic activation that may lead to a redistribution of membrane components to sites of new synapse formation. Thus, the growth of synaptic connections that accompanies long-term facilitation in *Aplysia* seems to be accompanied by a coordinated program of clathrin-mediated endocytosis, leading to the internalization of cell adhesion molecules.

Findings in other invertebrate systems and the mammalian brain have strengthened the notion that cell adhesion molecules may play a role in various forms of synaptic modification (reviewed by Doherty et al., 1995; Fields and Itoh, 1996; Martin and Kandel, 1996). For

example, in Drosophila, down-regulation of the cell adhesion molecule Fasciclin II, a homolog of vertebrate NCAM and *Aplysia* apCAM, is both necessary and sufficient for activity-dependent presynaptic outgrowth at the postembryonic nerve–muscle synapse (Davis et al., 1996; Schuster et al., 1996a, 1996b). These studies in *Aplysia* and Drosophila suggest down-regulation may be a general mechanism underlying the modulation of cell adhesion molecules during physiological forms of synaptic plasticity and growth, and raise two important and interrelated questions: (1) what are the subcellular and molecular mechanisms that lead to the down-regulation of adhesion molecules, and (2) how does this down-regulation signal synaptic growth?

We have begun to address these questions by examining the molecular steps that underlie the down-regulation of apCAM. Utilizing a highly efficient neuronal expression vector (Kaang et al., 1993), we have selectively overexpressed hemagglutinin epitope-tagged (HA-tagged) constructs of either the transmembrane or glycosylphosphoinositol-linked (GPI-linked) isoforms of apCAM in cultured sensory neurons. By combining thin-section electron microscopy with immunolabeling of gold-conjugated antibodies, we find that only the transmembrane form is internalized following exposure to 5-HT. Whereas both isoforms of apCAM have identical extracellular domains, the cytoplasmic tail of the transmembrane form provides a substrate for intracellular signal transduction and cytoskeletal interactions. Overexpression of epitope-tagged transmembrane constructs with specific deletions of or mutations in the cytoplasmic domain has allowed us to demonstrate further that the 5-HT-induced down-regulation and concomitant internalization can be blocked by removing either the entire cytoplasmic tail or just the spanning region of the intracellular domain containing a PEST sequence (thought to target degradation), or by simply substituting alanine for threonine in the two mitogen-activated protein kinase (MAPK) consensus sites. In addition, we find that injection of a specific inhibitor of MAPK into sensory neurons, which blocks long-term facilitation (Martin et al., 1997 [this issue of *Neuron*]), also blocks down-regulation and internalization of the endogenous form of apCAM. These data suggest that activation of the MAPK pathway is important for the internalization of apCAM and may represent one of the initial stages of learning-related synaptic growth in *Aplysia*. In a broader sense, our data and those of the preceding paper suggest that there are inhibitory constraints on memory storage at the membrane as well as in the nucleus, and that one function of the recruitment of MAPK may be to remove inhibitory constraints on memory storage that normally are active within the cell.

Results

Epitope Tagging of apCAM Variants and Their Expression in *Aplysia* Neurons

We modified the coding sequence of apCAMs (Mayford et al., 1992) by tagging them with the HA epitope (YPYDVPDYA) derived from hemagglutinin of the influenza virus, in order to monitor ectopic expression of apCAMs using a monoclonal anti-HA antibody, 12CA5. The viral epitope HA was inserted into a putative loop segment between β-strands B and C in the first immunoglobulin domain, to minimize possible structural distortion after insertion and to maximize accessibility of anti-HA antibody to the epitope (Ryu et al., 1990). The epitope sequence was incorporated by recombinant polymerase chain reaction (PCR) between Ser (residue 56) and Asp (residue 57), which is of equal distance from the highly conserved Cys (residue 50) within β-strand B and from Trp (residue 63) within β-strand C (Williams and Barclay, 1988). We also generated mutations in the cytoplasmic tail of the HA-tagged transmembrane isoform of apCAM to assess its function in 5-HT-regulated internalization. These mutations include the deletion of the entire cytoplasmic tail, the deletion of the PEST motif at the carboxy terminal, and point mutations in MAP kinase phosphorylation sites within PEST (Figure 1A). Each of the HA-tagged isoforms and transmembrane mutants was subcloned into the multiple cloning sites of the expression vector pNEXδ (Kaang et al., 1993), in order to express these apCAM variants ectopically by microinjection into *Aplysia* neurons.

A Western blot of protein extracts from *Aplysia* neurons microinjected with expression vectors containing HA-tagged apCAM cDNA variants was probed with anti-HA antibody 12CA5 (Figure 1B). Molecular weights of bands in a blot range from 107–144 kDa; these are higher than the calculated sizes, ranging from 84–102 kDa, based on the amino acid sequences of recombinant proteins. This property of gel migration was previously reported from native apCAMs, although the nature of the posttranslational modification of apCAM is not clear (Mayford et al., 1992). This indicates that these recombinant apCAMs were expressed and modified in the same way as native ones, without a serious degradative modification after microinjection-based gene transfer.

The subcellular localization of overexpressed apCAM isoforms was examined by immunofluorescence, using the Cy3-conjugated anti-HA antibody. The immunofluorescence data show that the *Aplysia* neurons expressing HA-tagged apCAM isoforms by microinjection form a ring-like structure around cell somata of sensory neurons, and also emit a strong fluorescence from neurites (data not shown), indicating that the recombinant apCAM isoforms were successfully targeted to the plasma membrane of the cell body and neurites after gene transfer.

5-HT Induces Internalization of the Transmembrane but Not the GPI-Linked Isoform of apCAM in Sensory Neurons

Within one hour of its application, 5-HT leads to a 50% decrease in the density of apCAM at the surface membrane of sensory neurons (Bailey et al., 1992). This partial down-regulation may be explained by some heterogeneity in internalization exhibited by the different isoforms of apCAM. As shown in Figure 1A, one of these isoforms is transmembrane in disposition, while the others are attached to the membrane by a GPI-linkage. To examine the specificity of the 5-HT-induced down-regulation of these different isoforms, we have utilized the pNEXδ expression vector (Kaang et al., 1993) to selectively overexpress HA-tagged constructs of either the transmembrane or large GPI-linked isoforms of apCAM in isolated sensory neurons. To localize each isoform at

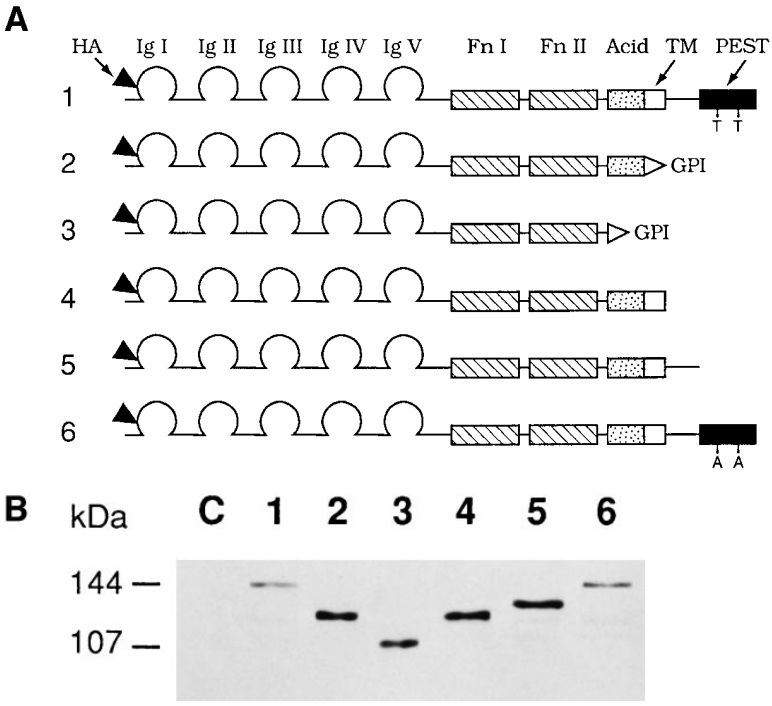

Figure 1. HA-Tagged apCAM Variants and Their Expression in *Aplysia* Neurons

(A) Schematic representation of the domain organization of apCAM isoforms and mutants used in this study. 1, HA-tagged transmembrane isoform (HA-apCAM-TM); 2, HA-tagged GPI-linked isoform (large); 3, HA-tagged GPI-linked isoform (small); 4, HA-tagged transmembrane isoform lacking the cytoplasmic tail; 5, HA-tagged transmembrane isoform lacking the PEST domains; 6, HA-tagged transmembrane isoform lacking putative MAP kinase phosphorylation sites. Ig, immunoglobulin domain of the C-2 type; Fn, fibronectin type III; TM, transmembrane domain; PEST, PEST domain; GPI, glycosylphosphoinositol attachment signal; Acid, glutamate-rich domain; HA, hemagglutinin epitope tag.

(B) Western blot analysis of various HA-tagged apCAM isoforms and mutants expressing in *Aplysia* neurons. The recombinant apCAM molecules seem to migrate slower than expected, probably because they contain a glutamate-rich acidic domain (except the smaller GPI-linked isoform) and undergo an unidentified posttranslational modification. C, negative control (no expression); 1, HA-tagged transmembrane isoform (HA-apCAM-TM) (144 kDa/102 kDa) (MW measured from gel mobility/MW based on amino acid sequence); 2, HA-tagged GPI-linked isoform (large) (130 kDa/88 kDa); 3, HA-tagged GPI-linked isoform (small) (107 kDa/84 kDa); 4, HA-tagged transmembrane isoform lacking the cytoplasmic tail (131 kDa/89 kDa); 5, HA-tagged transmembrane isoform lacking the PEST domains (136 kDa/94 kDa); 6, HA-tagged transmembrane isoform lacking putative MAP kinase phosphorylation sites (144 kDa/102 kDa).

the ultrastructural level, a mouse anti-HA antibody was used to recognize the HA epitope coupled with a gold-conjugated anti-mouse IgG antibody.

A summary of our results is shown in Figure 2. A 1 hr continuous exposure of 5-HT led to a 68% decrease in the density of gold-labeled complexes bound to the transmembrane form at the surface membrane ($9 \pm 0.5/\mu m$, $n = 8$, versus control $28 \pm 3/\mu m$, $n = 7$, $P < 0.01$) and to a 26-fold increase in their internalization ($53 \pm 4\%$, versus $2 \pm 0.7\%$, $P < 0.01$). By contrast, 5-HT had no effect on either the surface distribution ($27 \pm 2/\mu m$, $n = 7$, versus $26 \pm 2.4/\mu m$, $n = 6$) or internalization ($2 \pm 0.5\%$, versus $2.2 \pm 0.9\%$) of the GPI-linked isoform. These population differences are reflected in the examples of the raw data illustrated in Figure 3. Figure 3A represents an example of a neurite of a sensory neuron expressing the GPI-linked isoform of apCAM and shown following a 1 hr exposure to 5-HT. Virtually all of the gold is on the surface membrane with little inside the cell, despite an apparent robust, general endocytic activation in response to 5-HT, as indicated by numerous internal membranous profiles. In striking contrast to the lack of down-regulation of the GPI-linked isoform, Figure 3B illustrates the dramatic effect the same exposure to 5-HT has on the transmembrane isoform of apCAM, removing most of it from the surface membrane, resulting in heavy intracellular accumulations of gold complexes within putative endocytic compartments.

As was the case in the initial study utilizing a monoclonal antibody that recognizes all of the apCAM isoforms, internalization of the transmembrane form is initiated at coated pits and proceeds through a similar degradative pathway, including early, smooth-surfaced vesicles, sorting endosomes, and multivesicular bodies, and eventually can be found within mature lysosomes in the cell body. An example of one of the later endosomal compartments containing the internalized transmembrane isoform is illustrated in Figure 4A, which depicts heavy accumulations of gold within a large multivesicular body. A better appreciation of the extent of the 5-HT-induced endocytic activation and the magnitude of the internal labeling is shown in Figure 4B, where an extensive field of polymorphic tubular and vesicular profiles can be seen, virtually all of which contain internalized gold bound to the transmembrane isoform of apCAM.

Deletion of the Cytoplasmic Tail or the Spanning Region Containing PEST Blocks Internalization of apCAM

The selective internalization of the transmembrane isoform highlights the potential regulatory significance of its intracellular domain, which contains a prominent PEST sequence (PEST sequences are enriched in proline, glutamic acid, serine, and threonine residues; are often found in proteins with short half-lives; and are thought to be a signal targeting degradation) (Rechsteiner and Rogers, 1996) and has two putative consensus sites for phosphorylation by MAPK (Mayford et al., 1992; D. Michael, personal communication). To begin our examination of which portions of the apCAM molecule might be important for triggering internalization, and which may play a role in targeting degradation, we used two epitope-tagged deletion mutants: a truncated form lacking the intracellular domain, and a mutant that

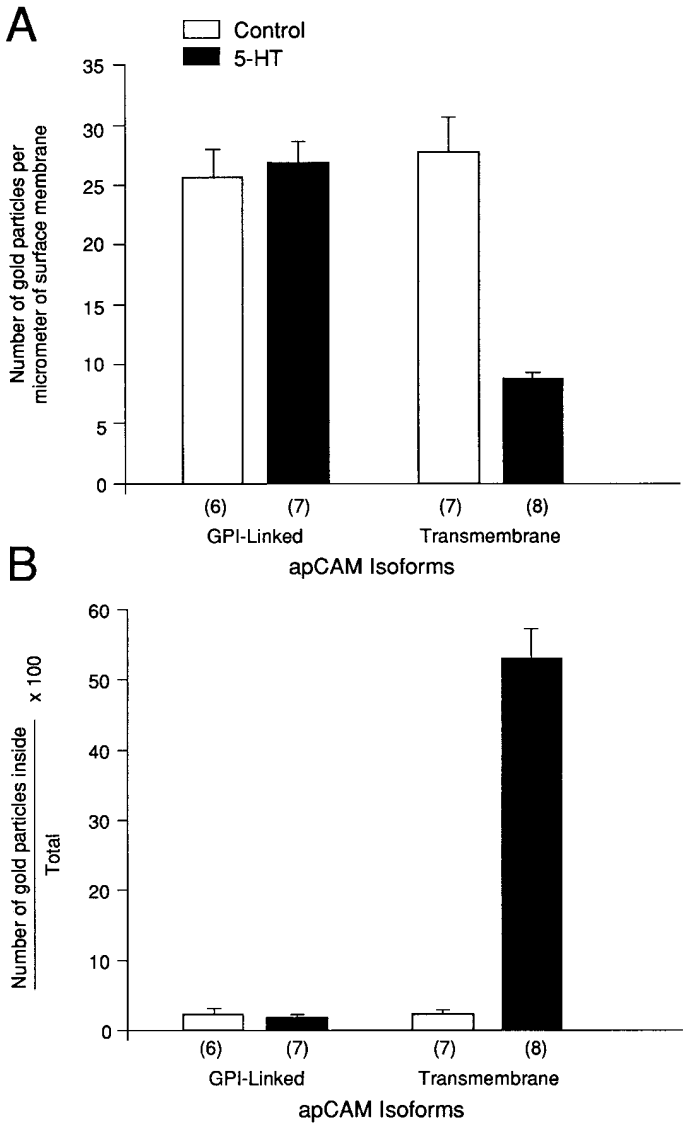

Figure 2. 5-HT Induced Down-Regulation of the Transmembrane Isoform of apCAM

Selective down-regulation of the transmembrane isoform of apCAM induced by a 1 hr exposure to 5-HT at the sensory neuron membrane.

(A) Surface density of transmembrane and GPI-linked isoforms of apCAM as determined by the distribution of gold-conjugated Ab complexes bound to each form.

(B) Density of each apCAM isoform inside the cell. Each bar represents the mean ± SEM.

lacked a spanning region that contains the PEST sequences and two MAPK consensus sites. The effect of 5-HT on these two deletion constructs is summarized in Figures $5A_1$ and $5B_1$. Down-regulation at the surface membrane and internalization are completely blocked, not only by removing the entire cytoplasmic tail (28 ± 4/μm; 1 ± 0.1%, n = 5, $P < 0.01$), but also just as effectively by deletion of the PEST sequences (33 ± 4/μm; 3 ± 0.7%, n = 3, $P < 0.01$).

Mutation of MAPK Phosphorylation Sites Blocks Internalization of apCAM

Similar to the internalization of apCAM, MAPK activity is enhanced in sensory neurons of *Aplysia* by repeated treatments of 5-HT and by elevated levels of intracellular cAMP (D. Michael, personal communication; Martin et al., 1997). Since removal of the PEST sequences, which contain the two MAPK consensus sites, blocks the 5-HT-induced down-regulation of apCAM, we also examined if simply altering the MAPK sites themselves would affect internalization. To accomplish this, the two putative MAPK phosphorylation sites at threonine 889 and threonine 923 were mutated to alanine. We find that by substituting alanine for threonine in the MAPK sites, we can block both the 5-HT-induced down-regulation at the surface membrane (35 ± 4/μm) and the concomitant internalization of the transmembrane form (1 ± 0.7%, n = 5, $P < 0.01$; Figures $5A_1$ and $5B_1$).

PD098059, A Pharmacological Inhibitor of MEK1, Also Blocks Internalization of the Endogenous Form of apCAM

The role of the MAPK cascade in the internalization of the endogenous form of apCAM was examined by using PD098059 (Warner-Lambert), a specific pharmacological inhibitor of MEK1, the kinase immediately upstream of MAPK (Alessi et al., 1995). PD098059 has been found to selectively block long-term facilitation, without affecting either short-term facilitation or basal synaptic transmission (Martin et al., 1997), as well as to block basal

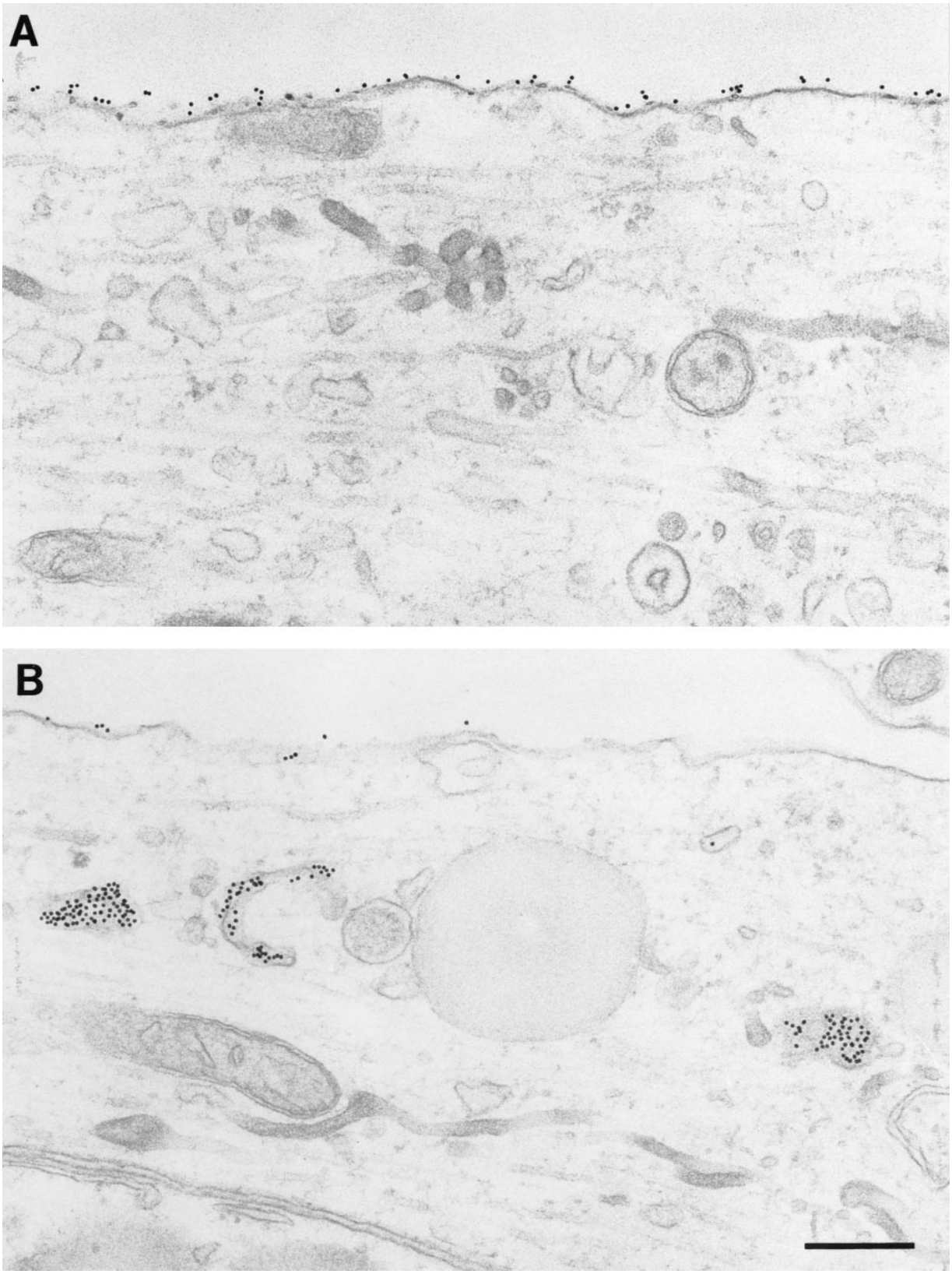

Figure 3. Differential Down-Regulation of the GPI-Linked versus Transmembrane Isoforms of apCAM

(A) Neurite of a sensory neuron expressing the GPI-linked isoform of apCAM following a 1 hr exposure to 5-HT. Note virtually all the gold complexes remain on the surface membrane with none inside, despite a robust 5-HT-induced activation of the endosomal pathway leading to significant accumulations of internal membranous profiles.

(B) Neurite of a sensory neuron expressing the transmembrane isoform of apCAM following a 1 hr exposure to 5-HT. In contrast to the lack of down-regulation of the GPI-linked isoform, 5-HT has a dramatic effect on the transmembrane isoform of apCAM removing most of it from the surface membrane, resulting in heavy accumulations of gold complexes within presumptive endocytic compartments. Scale for (A) and (B) = 0.25 μm.

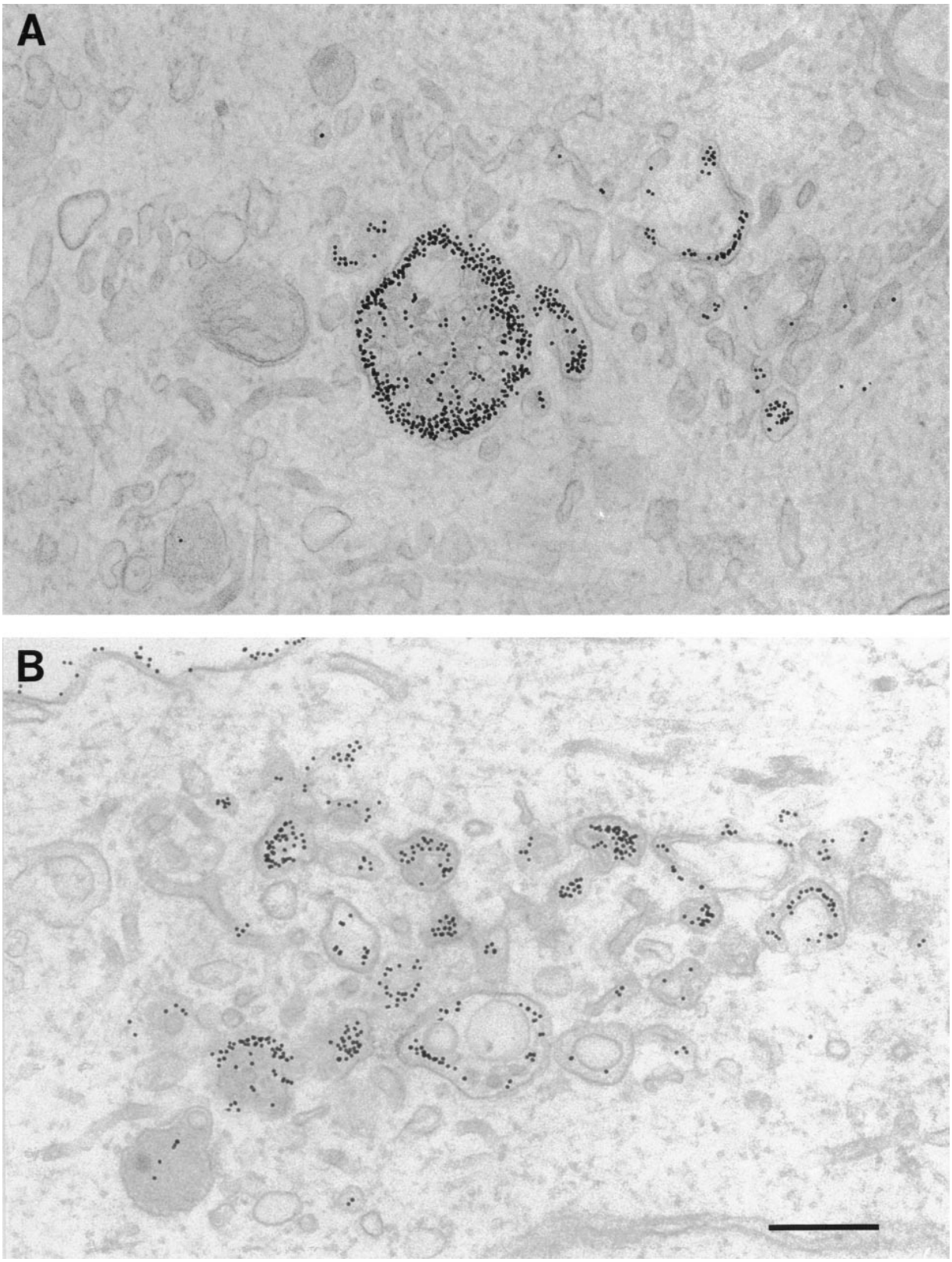

Figure 4. Intracellular Fate of the Transmembrane Isoform of apCAM

(A) The internalized transmembrane isoform of apCAM can be found in a variety of putative later endosomal compartments including multivesicular bodies.

(B) The extent of the endocytic response to a 1 hr exposure of 5-HT and the relative magnitude of the internal labeling of the transmembrane isoform of apCAM are shown. Note the large field of polymorphic tubular and vesicular profiles, virtually all of which contain gold complexes bound to the transmembrane form of apCAM. Scale for (A) and (B) = 0.25 μm.

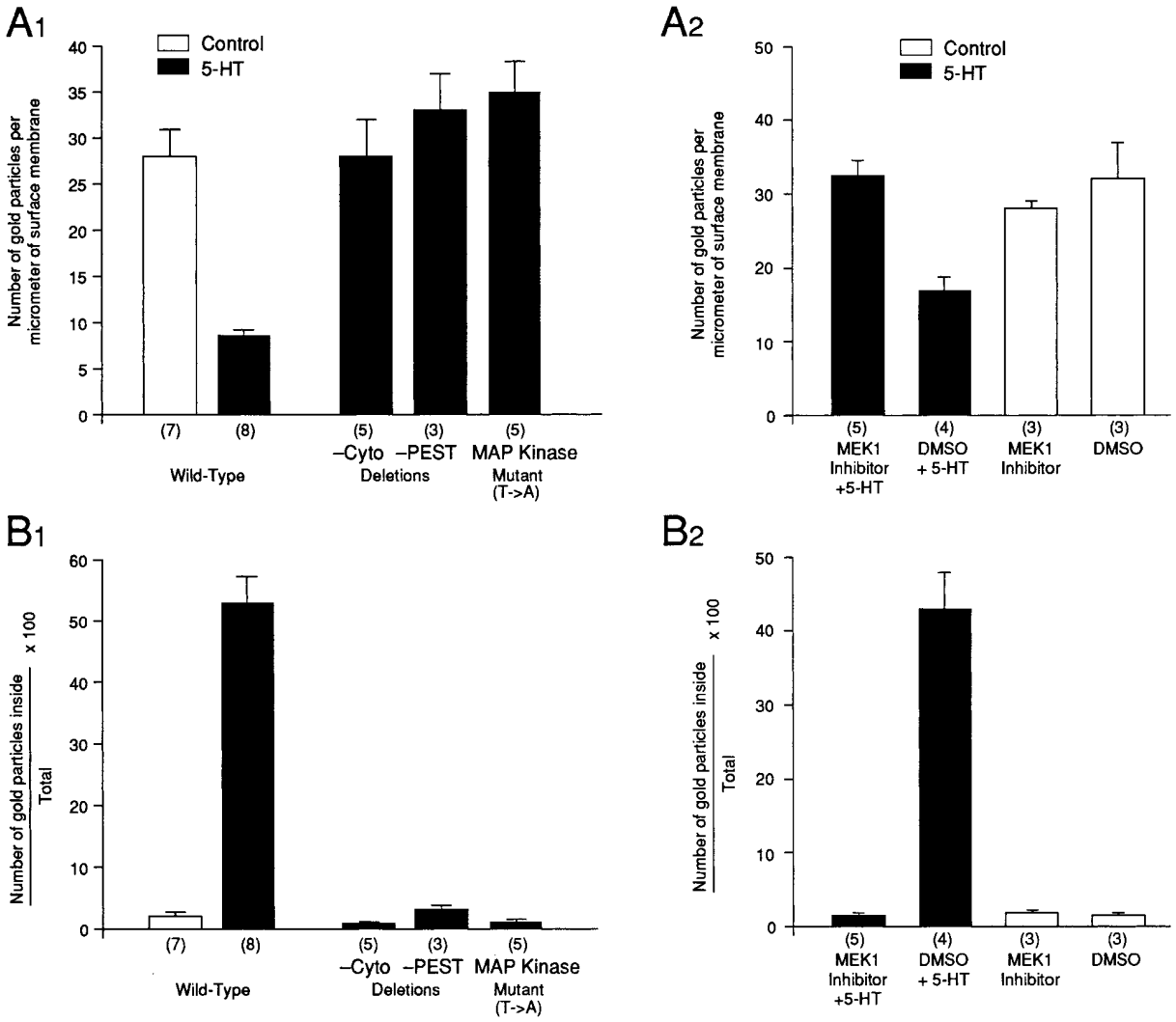

Figure 5. Inhibiting MAPK Blocks the Internalization of apCAM

(A_1 and B_1) Effects of 5-HT on three transmembrane isoform mutants. Removal of the entire cytoplasmic tail, just the spanning region containing the PEST sequences, or the simple substitution of alanine for threonine in the two MAPK phosphorylation sites at 889 and 923 effectively blocks both (A_1) down-regulation at the surface membrane and (B_1) internalization of the transmembrane isoform of apCAM. Each bar represents the mean ± SEM.

(A_2 and B_2) PD098059, an inhibitor of the MAPK pathway, also blocks down-regulation of the endogenous form of apCAM. Intracellular injection of PD098059 (10 μM in 0.01% DMSO) into sensory neurons 2 hr before a 1 hr exposure to 5-HT blocks both (A_2) down-regulation and (B_2) internalization of the endogenous form of apCAM. Each bar represents the mean ± SEM.

MAPK activity in *Aplysia* sensory neurons (D. Michael, personal communication). Thus, the functional effect of PD098059 on sensory neurons appears to be specific and mediated by blocking the phosphorylation of intracellular sites, rather than by means of a more global and nonspecific action, such as the shutting down of basic cellular processes that would be toxic to the cell. We find that injection of PD098059 (10 μM in 0.01% DMSO) into sensory neurons 2 hr before long-term training with 5-HT effectively blocks both down-regulation at the surface membrane (32.4 ± 2/μm) and the internalization of a gold-conjugated monoclonal antibody specific to apCAM (1.5 ± 0.4%, n = 5, $P < 0.01$) when compared with cells injected with the buffer vehicle (DMSO) alone and similarly treated with 5-HT (16.8 ± 1.8/μm; 43 ± 5%, n = 4). In the absence of 5-HT, injection of either PD098059 or DMSO had no effect on down-regulation (28 ± 1.3/μm, n = 3; 32 ± 5.4/μm, n = 3) or internalization (1.9 ± 0.5%; 1.5 ± 0.5%, Figures $5A_2$ and $5B_2$).

Discussion

An increasing body of evidence from both invertebrates and vertebrates suggests modulation of cell adhesion molecules is important for both developmental and learning-related synaptic plasticity (reviewed by Rutishauser, 1993; Doherty et al., 1995; Fields and Itoh, 1996; Martin and Kandel, 1996). During development of the nerve–muscle synapse in the chick, removal of the cell surface carbohydrate, polysialic acid, from NCAM (a

procedure functionally equivalent to down-regulation) inhibits normal branching and proper axonal guidance (Landmesser et al., 1992; Tang et al., 1992). Subsequent studies in the vertebrate central nervous system have provided additional support for the notion that cell adhesion molecules may also regulate long-term synaptic plasticity in the adult. For example, in the rat hippocampus, perturbation of the neural cell adhesion molecules L1 and NCAM reduces long-term potentiation in CA1 neurons (Lüthl et al., 1994). In addition, Itoh et al. (1995) have found the expression of cell adhesion molecules in hippocampal culture can be altered by changes in neuronal activity. Moreover, the memory for passive avoidance, a form of associative learning in the chick that correlates with the expression of NCAM and L1, is blocked by intracerebral injection of antibodies against NCAM or L1 (Rose, 1995).

Activity-dependent synaptic sprouting in Drosophila similarly requires changes in the expression of the cell adhesion molecule Fasciclin II, a homolog of *Aplysia* apCAM and vertebrate NCAM (Davis et al., 1996; Schuster et al., 1996a, 1996b). Fasciclin II is expressed both pre- and postsynaptically at the Drosophila nerve–muscle synapse, and expression at both sites is required for stabilization following synaptogenesis. Once the synapse is stabilized, activity-dependent structural plasticity is accompanied by a 50% decrease in presynaptic levels of Fasciclin II, similar to the down-regulation of apCAM in *Aplysia*. This decrease in Fasciclin II at the synapse is both necessary and sufficient for presynaptic sprouting; mutants that decrease Fasciclin II by 50% lead to synaptic sprouting, while transgenes that maintain presynaptic Fasciclin II at elevated levels prevent the activity-dependent structural changes. Consistent with these findings, the recently identified Drosophila learning mutant, *Volado*, has a mutation in the cell adhesion molecule α-integrin (R. L. Davis, personal communication).

This generality raises the question: what are the intracellular signaling pathways whereby the down-regulation of cell adhesion molecules occurs? The work in *Aplysia* indicates 5-HT, a modulating neurotransmitter that is important for learning, can stimulate receptor-mediated endocytosis and lead to the rapid internalization of an NCAM-related cell adhesion molecule, a process that appears to be an early step leading to the growth of new synaptic connections associated with long-term facilitation.

How is the 5-HT-induced internalization of apCAM achieved? To address this question, we have selectively overexpressed specific, epitope-tagged deletion and mutant constructs of apCAM in isolated sensory neurons. We find that the cytoplasmic domain of this cell adhesion molecule is required for signaling endocytosis. The endocytosis of constitutively internalized receptors, like low density lipoprotein or transferrin, occurs via clathrin-coated pits and is mediated by a direct interaction between the cytoplasmic tail and intracellular adaptor molecules that are components of the clathrin coat, a process that depends upon a specific tyrosine-containing motif in the cytoplasmic tail of the receptor (reviewed by Robinson, 1994). Interestingly, unlike the low

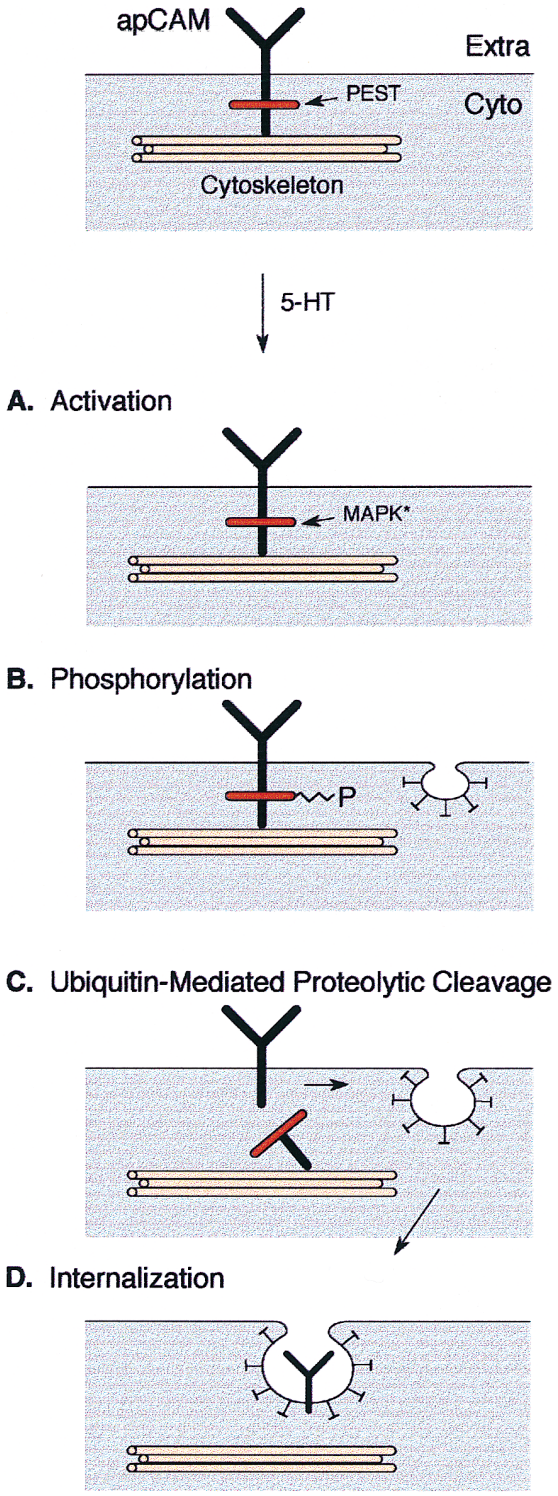

Figure 6. Speculative Model of Molecular Steps Underlying the 5-HT-Induced Internalization of apCAM

Repeated or prolonged applications of 5-HT or elevated levels of cAMP activates the MAPK pathway leading to the phosphorylation of the cytoplasmic tail of apCAM. This phosphorylation, either by itself or followed by a proteolytic change (perhaps mediated by ubiquitin hydrolase), could alter the conformation of the intracellular domain of apCAM or its interactions with cytoskeletal elements, such that the cell adhesion molecules could now be more easily localized to coated pits and subsequently internalized.

density lipoprotein and transferrin receptors, the intracellular domain of apCAM lacks a typical tyrosine-containing internalization sequence and, indeed, is not constitutively internalized in the absence of 5-HT (Bailey et al., 1992). Nevertheless, our data suggest the site of specificity of 5-HT's effects is at the cytoplasmic tail of the transmembrane form.

Given that MAPK is important for the down-regulation of apCAM, what is its role in internalization? A common signal for internalization is proteolytic cleavage. This is consistent with the prominent PEST sequences and raises the possibility that apCAM could also be a target for ubiquitin-mediated proteolysis, since C-terminal ubiquitin hydrolase is one of the early effectors turned on by 5-HT in *Aplysia* sensory neurons (Hegde et al., 1997). In fact, two studies have suggested a connection between endocytosis and the ubiquitin pathway. Hicke and Riezman (1996) demonstrated that ubiquitination of a yeast plasma membrane receptor signals its endocytosis, and Nelson and Lemmon (1993) found that overexpression of ubiquitin rescues clathrin deficiency. Moreover, Fazelli et al. (1994) have shown that LTP in the hippocampus is accompanied by a concomitant elevation of soluble NCAM and increased proteolytic activity, suggesting that NCAM is also proteolytically removed from the surface membrane in an activity-dependent fashion. One of the consequences of MAPK phosphorylation or proteolytic cleavage might be an alteration in the conformation of the cytoplasmic domain of apCAM or its interaction with cytoskeletal elements, so that these cell adhesion molecules could now be more easily localized to coated pits and subsequently be more susceptible to internalization (Figure 6). Aspects of this scheme may be similar to what is thought to occur with the epidermal growth factor (EGF) receptor, where phosphorylation of the cytoplasmic tail has been shown to be a prerequisite for proteolytic cleavage (Honegger et al., 1990).

As indicated by Martin et al. (1997), activation of the MAPK pathway can, in addition, function at sites other than the surface membrane of sensory neurons. Following repeated presentations of 5-HT, apMAPK undergoes nuclear translocation, and thus becomes strategically positioned for potential modulation of transcriptional regulators essential for the consolidation of long-term facilitation. In fact, CREB-2, a repressor of long-term facilitation, has no cAMP-dependent protein kinase (PKA) phosphorylation sites but has prominent MAPK sites (Bartsch et al., 1995). Thus, MAPK might act both at the surface membrane and in the nucleus to remove inhibitory constraints on long-term memory storage.

The significance of the differential down-regulation of the GPI-linked and transmembrane isoforms of apCAM, especially as it relates to synapse formation, remains unknown. Our data suggest that there may be fundamentally different mechanisms for sorting these proteins and that they may have different functions as well as different adhesive capabilities. The finding that 5-HT leads to the rapid down-regulation of only one isoform of apCAM (the transmembrane isoform) and not the others (the GPI-linked isoforms) raises the interesting possibility that learning-related synaptic growth in the adult may be initiated by an activity-dependent recruitment of specific isoforms of adhesion molecules, similar to the modulation of cell surface receptors during the fine-tuning of synaptic connections in the developing nervous system. One consequence of isoform recruitment is that it would allow neuronal activity to regulate the surface expression of each isoform, a process that might

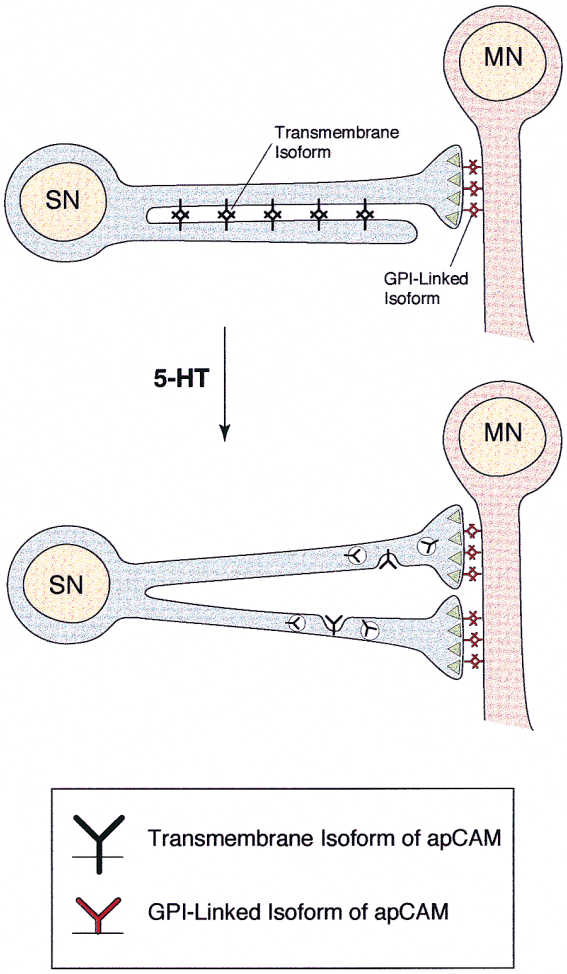

Figure 7. Regional Specific Down-Regulation of the Transmembrane Isoform of apCAM

This model is based on the assumption that the relative concentration of the GPI-linked versus transmembrane isoforms of apCAM is highest at points of synaptic contact between the sensory neuron and motor neuron and reflects the results of studies done in dissociated cell culture. Thus, previously established connections might remain intact following exposure to 5-HT since they would be held in place by the adhesive, homophilic interactions of the GPI-linked isoforms and the process of outgrowth from sensory neuron axons would be initiated by down-regulation of the transmembrane form at extrasynaptic sites of membrane apposition. In the intact ganglion, the axons of sensory neurons are likely to fasciculate not only with other sensory neurons but also with the processes of other neurons and perhaps even glia. One of the attractive features of this model is that the mechanism for down-regulation is intrinsic to the sensory neurons. Thus, even if some of the sensory neuron axonal contacts in the intact ganglion were heterophilic in nature, i.e., with other neurons or glia, we would still expect the selective internalization of apCAM at the sensory neuron surface membrane at these sites of heterophilic apposition to destabilize adhesive contacts and to facilitate disassembly.

take on additional functional significance if these surface molecules were distributed differentially along the three-dimensional extent of the neuron.

At the present time, the precise spatial distribution of the native transmembrane and GPI-linked isoforms of apCAM along the sensory neuron axonal arbor is not known. Using a monoclonal antibody that recognizes all of the apCAM isoforms, Zhu et al. (1995) have shown that repeated presentations of 5-HT to sensory-motor neuron cocultures increase the relative levels of apCAM at preexisting sensory neuron varicosities. These findings, combined with the present study's demonstration that 5-HT produces a selective down-regulation of the transmembrane form, suggest that the relative concentration of the GPI-linked versus the transmembrane forms is highest at sites of synaptic contact. Thus, the distribution of the two isoforms of apCAM may occur in such a fashion that the activity-independent GPI-linked forms might serve primarily, if not exclusively, to stabilize synaptic contacts, whereas the activity-dependent transmembrane forms might primarily or exclusively stabilize only contacts between fasciculated axonal processes. This would suggest that previously established synaptic connections remain intact following exposure to 5-HT, and that the process of outgrowth from sensory neuron axons is initiated by down-regulation of the transmembrane form at extrasynaptic sites of membrane apposition (Figure 7). The availability of deletion mutants that do not undergo down-regulation may now allow us to explore these possibilities directly, and should provide additional mechanistic insights about the precise role that the endocytic response and resultant internalization of a selective isoform of apCAM may play in the initiation of learning-related synaptic growth.

Experimental Procedures

DNA Constructions Using PCR

Generation of HA-Tagged Transmembrane Isoform of apCAM (HA-apCAM-TM)

Amplification from d19 template (Mayford et al., 1992) with a sense primer 1 (5′-tccc*aagcTT*CCTGAATGCAACACT-3′, HindIII site is italicized) and an antisense primer 1 (3′-CCCGGAGACGTTGGAGG*ATG GGTATGCTACAAGGTCTAATGCGGC*-5′, HA epitope coding sequence is italicized) generated a 0.23 kb fragment (denaturation at 94°C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 1 min, 20 cycles). Next, a second fragment (0.28 kb) was produced by amplification (the same PCR condition, except annealing at 54°C) from the same template with a sense primer 2 (5′-CC*TACCCATACG ATGTTCCAGATTACGCC*GACCAGGTCACGCTCAA-3′, HA epitope coding sequence is italicized) and an antisense primer 2 (3′-ACTGTA GGACCCGCCCC-5′) (denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min, 20 cycles). These two overlapping fragments were mixed, and recombinant PCR (denaturation at 94°C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 1 min, 30 cycles) was performed with the sense primer 1 and antisense primer 2 to generate a 0.49 kb fragment; this product was then digested with HindIII and TfiI to release a recombinant fragment (0.38 kb) containing the N-terminal portion of HA-tagged apCAM. The remaining C-terminal DNA fragment (2.5 kb) of apCAM was rescued by cutting the d19 construct with TfiI and StuI. These two fragments (0.38 kb and 2.5 kb), encompassing the whole sequence of the transmembrane isoform of apCAM, were ligated into HindIII-SmaI linearized pNEXδ, a neuronal expression vector (Kaang et al., 1993). This DNA construct (HA-apCAM-TM) was used to express by microinjection the HA-tagged transmembrane isoform of apCAM in *Aplysia* neurons.

HA Tagging of the GPI-Linked Isoform

The C-terminal DNA sequences of the small or large GPI-linked isoforms was amplified by PCR (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, 25 cycles) from d12 (the small isoform) or d15 (the large isoform) templates (Mayford et al., 1992) with a sense primer 3 (5′-CTCGCCCATTAAGC ACT-3′) and an antisense primer (3′-ACTGGAAGTGTAAGAG*Ccatgg* ggc-5′, KpnI site is italicized), generating 0.37 kb or 0.50 kb, respectively. These fragments were cut with BamHI and KpnI to release 0.27 kb or 0.40 kb; these fragments were substituted for a 0.81 kb fragment within HA-apCAM-TM, thus creating HA-tagged small or large GPI-linked isoforms, respectively.

Deletion of the Entire Cytoplasmic Tail from HA-apCAM-TM

Amplification from d19 template with the sense primer 3 and an antisense primer (3′-AACAGTGGTTCTTCTTC*attatccatgg*ggc-5′, stop codons and KpnI site are italicized) generated a 0.50 kb fragment (denaturation at 94°C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 1 min, 25 cycles). After cutting with BamHI and KpnI, the released 0.40 kb fragment substituted for a 0.81 kb fragment within HA-apCAM-TM, thereby creating a mutant lacking the entire cytoplasmic tail, except three lysine residues juxtaposing the α-helical transmembrane domain.

Deletion of the PEST Domain from HA-apCAM-TM

To generate a mutant construct lacking the PEST domain within the transmembrane isoform, HA-apCAM-TM was cleaved with BbsI, and the resulting small fragment (0.15 kb) was replaced by the short double-stranded oligonucleotides generated by annealing two primers (5′-TGTG*TAATGA*AGACT-3′ and 3′-*ATTACT*TCTGAGGGG-5′, stop codons are italicized). This created the PEST deletion mutant, which lacks amino acid sequences from the residue Lys (860) to the C-terminus.

Point Mutagenesis of PEST Consensus Sequence in HA-apCAM-TM

Two potential MAP kinase phosphorylation sites (amino acids 887–890 and 921–924 sequence: PETP) were mutated by replacing two threonine residues (889 and 923) with alanine through three rounds of recombinant PCR. A 0.72 kb fragment (#1) was amplified from the HA-apCAM-TM template with a sense primer 3 and an antisense primer 3 (3′-GACTCTGGTTCGGTCTC*C*GGGGCCGGC-5′, italicized base C is mutated from base T) (denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min, 25 cycles). A 0.12 kb fragment (#2) was amplified from the same template with a sense primer 4 (5′-GAGACCAAGCCAGAGGCCCCGGCCGAGCCA CCA-3′, italicized base G is mutated from base A) and an antisense primer 4 (3′-TAGTTCTAGTGGGGTCTCCGGGGGC-5′, italicized base C is mutated from base T) (denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min, 25 cycles). A 0.16 kb fragment (#3) was amplified from the same template with a sense primer 5 (5′-CACCCCAGAG*G*CCCCCGAGAAGCCTGAG-3′, italicized base G is mutated from base A) and an antisense primer 5 (3′-GGTCAACCAGACCACAG-5′, this sequence resides within the expression vector pNEXδ) (denaturation at 94°C for 1 min, annealing at 42°C for 1 min, extension at 72°C for 1 min, 25 cycles). The overlapping fragments #2 and #3 were mixed and recombinant PCR (denaturation at 94°C for 1 min, annealing at 42°C for 1 min, extension at 72°C for 1 min, 30 cycles) was carried out with the sense primer 4 and antisense primer 5 to generate a 0.26 kb fragment (#4). Next, the overlapping fragments #1 and #4 were mixed, and the final recombinant PCR (denaturation at 94°C for 1 min, annealing at 42°C for 1 min, extension at 72°C for 1 min, 30 cycles) was performed with the sense primer 3 and antisense primer 5 to yield a 0.97 kb fragment (#5). This fragment #5 was digested with BamHI and KpnI, and the resulting 0.80 kb fragment was used to substitute for the corresponding fragment in HA-apCAM-TM, thereby creating an HA-apCAM-TM mutant, where the two threonine residues in MAPK phosphorylation sites are all mutated to alanine.

DNA Sequencing

The sequences of all mutations and any misincorporation during PCR amplification were checked by the dideoxy DNA sequencing using a T7 sequenase system (USB).

Sensory Cell Cultures

Aplysia sensory neurons were isolated from pleural ganglia dissected from adult animals (70–120 g), as previously described

(Schacher and Proshansky, 1983). Individual cells were removed from the ganglia with a segment (200–600 μm) of their axons intact and placed in dissociated cell culture. Each culture dish contained approximately 20–50 sensory cells. Cultures were allowed to grow for 5 days to reach a stable level before each experiment.

Microinjection of Plasmid DNA into *Aplysia* Neurons

Microinjection of various DNA constructs (1 mg/ml DNA) dissolved in a buffer containing 0.1% fast green, 10 mM Tris-Cl (pH 7.3), and 100 mM NaCl was performed into *Aplysia* ganglionic neurons or cultured neurons by applying positive air pressure, as previously described (Kaang et al., 1992; Kaang, 1996). The impedance of the microcapillaries used in microinjection was 5–10 MΩ when they were filled with 3 M KCl. Microinjected cells were incubated at 18°C for 18–24 hr and used for Western blotting, immunocytochemistry, and immunogold labeling.

Western Blotting

Western blotting was performed with ganglionic lysates. Each ganglion containing 20 neurons microinjected with a specific DNA construct was lysed in 50 μl of boiling SDS gel sample buffer. Twenty microliters of each lysate was subjected to 7% SDS–PAGE, and proteins were subsequently transferred onto the Hybond ECL filter (Amersham) using the semidry electrophoretic transfer (Harlow and Lane, 1988). The filter was incubated with mouse monoclonal anti-HA Ab 12CA5 (BABCO, CA), diluted 1:2500, followed by horseradish peroxidase-conjugated goat anti-mouse IgG Ab (Pierce), diluted 1:5000. Immunoreactivity was detected with a chemoluminescence system (ECL, Amersham).

Immunocytochemistry

Cultured sensory cells were microinjected with each apCAM isoform DNA. The cultures were incubated 18 hr after microinjection and then fixed with 2% paraformaldehyde, 0.25% glutaraldehyde in phosphate-buffered saline for 10 min. The fixative was quenched with 50 mM NH_4Cl. After blocking the nonspecific binding by preincubating cells with 10% goat serum in phosphate-buffered saline, the cells were incubated with the Cy3-conjugated anti-HA Ab 12CA5, diluted at 1:150 in blocking solution. Anti-HA Ab 12CA5 was conjugated with Cy3 (Biological Detection Systems, Inc.), following the instruction manual.

Gold Labeling of Cells and 5-HT Application

The monoclonal anti-HA Ab 12CA5 and anti-apCAM Ab 4E8 (BABCO, CA) were purified from ascites by fluid affinity chromatography with protein A. Rabbit anti-mouse IgG2b Ab (Zymed) and the monoclonal Ab 4E8 were conjugated with 10 nm colloidal gold (AuroBeads, Amersham), following the instruction manual. Gold-conjugated antibodies were adjusted to a final OD (520 nm) of 10. The monoclonal anti-HA Ab 12CA5 was applied at 1:100 dilution to the sensory cells microinjected with various plasmid DNAs for 1 hr at room temperature. After washing with culture medium, cells were incubated with gold-conjugated anti-mouse IgG2b, diluted at 1:50. After rinsing to remove unbound gold-conjugated Ab, 5-HT (Sigma, creatine sulfate, 2–5 μM) was added for 1 hr continuous exposure at room temperature (Bailey et al., 1992; Mayford et al., 1992). In experiments assessing the effects of PD098059 (Warner-Lambert), cells were incubated with gold-conjugated monoclonal Ab 4E8 for 1.5 hr before rinsing and subsequent 5-HT application for 1 hr at room temperature.

Electron Microscopy and Quantitation of Gold Distribution

Cell cultures were rinsed briefly with hemolymph-free culture medium and rapidly fixed in place on poly-L-lysine-coated Aclar 33c coverslips, following a three-step protocol previously described (Bailey et al., 1992). Briefly, this included an initial fixation by slow perfusion with a trialdehyde solution containing 1% paraformaldehyde, 1% acrolein, 2.5% glutaraldehyde, and 2.5% dimethylsulfoxide with 10% sucrose plus $CaCl_2$ (0.05%) in 0.1 M cacodylate buffer (pH 7.4). After 1 hr at room temperature, this fixative was replaced with 2% paraformaldehyde and 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 16–20 hr at 4°C. The cultures were treated with 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) for 1 hr at room temperature. After the cultures were embedded in Epon 812, serial thin sections (50–100 sections per block) were cut parallel to the substrate surface, stained with lead and uranyl acetate, and photographed with a Phillips 301 electron microscope. Random regions of immunogold-labeled thin sections were sampled by taking micrographs at regularly spaced intervals throughout the neuritic arbor of the sensory neurons. Cell bodies were not included in the sample field for quantitative analysis. Since the transmembrane isoform was found heavily concentrated in numerous lysosomes within the cell bodies of sensory neurons following exposure to 5-HT, the actual number for internalization is likely to be much higher. Enlargements of the micrographs (80,000×) were quantitatively analyzed with a blind procedure. To quantitate the gold-labeled complexes, the micrographs were mounted on a Bioquant II digitizing tablet (R & M Biometrics, Inc., Nashville, TN) interfaced with an Apple IIe microcomputer, and the linear extent of surface membrane was obtained by digitized tracing. The total number of gold particles along the surface membrane was counted for each micrograph. An internalization index was computed by dividing the total number of gold particles inside the cell by the total number of gold particles for each micrograph. Data from each cell constituted one score or ratio.

Analysis of Data

All data are represented as the mean change ± SEM. A one-way analysis of variance and Newman-Keuls' Multiple Range Test were used to determine the statistical significance of all morphological data sets.

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